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Methods for pH Determination in Human Erythrocytes

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Summary: The methods described in the literature for pH determination in human erythrocytes, i.e. the direct potentiometric measurement of haemolysed erythrocytes and the 5,5-dimethyl-2,4-oxazolidinedione (DMO) method, were examined and compared. In spite of careful optimization of the experimental technique, a statistically significant difference of a few hundredths of a pH unit remained between the results of the two methods. The calculation of the cellular water space of the erythrocytes is suggested as a possible reason for this difference; in the DMO method this leads to uncertainty in the determination of the DMO concentration and therefore in the derived intracellular pH values. For clinical use, the direct pH measurement of haemolysed erythrocytes, using the technique described here, is recommended.

Introduction

Three different methods are commonly used for the determination of the pH value in erythrocytes (1). Firstly the pH value can be measured directly by potentiometry ("potentiometric method"), if the erythrocytes are haemolysed after separation from the plasma (2). It is also possible to determine the pH value on the basis of the pH-dependent partition of weak electrolytes between cells and plasma, e.g. as in the "DMO method", where the 5,5-dimethyl-2,4-oxazolidinedione (DMO) acts as a weak acid (3). Finally, the pH-dependent nuclear magnetic resonance spectra of specific compounds within the intact blood cells can be measured ("NMR method") (4). The DMO method is, like all related methods, very time-consuming, because other analyses are required in addition to the concentration determinations. The NMR method requires calibration by potentiometric pH determination, and furthermore involves the use of NMR apparatus. Therefore, only the direct potentiometric method is suitable for clinical purposes. The use of this method as a matter of routine makes appropriate control necessary. For checking the pH determination of the haemolysate the IFCC reference method (5) developed for whole blood can be used. This does not detect specimen-induced errors, such as those that may arise when collecting the erythrocytes. For a comprehensive check, an independent method should therefore be employed, and the DMO method is suitable for this purpose. Both the potentiometric and the DMO method can be carried out simultaneously on the same specimen material, without the results being subject to the same sources of error. When the DMO method was first introduced for measuring the pH value of erythrocytes, only a few comparative studies with the potentiometric method were carried out, and these did not show a satisfactory agreement (6, 7). Meanwhile, the analytical techniques for both methods have undergone further development. In the DMO method, the use of ^{14}C-labeled DMO with measurement by liquid scintillation spectrometry in particular has considerably increased the specificity and sensitivity. The method permits the direct DMO determination in cell material, whereas with the older UV spectrometric method the intracellular concentration of DMO had to be derived from the difference of whole blood and plasma concentrations. In the potentiometric method the reduction of the amount of specimen and the availability of largely gas-tight and temperature-resistant collecting tubes (monovette) have improved the reliability.

The present study examines whether identical results can be achieved with the two methods, using currently available technology and optimal test conditions, and thus whether the DMO method is suitable for checking the potentiometric pH determination in human erythrocytes.

Methods

Cell material, DMO incubation and haemolysate preparation

Venous donor blood is collected in a 25 ml monovette (serum monovette from Sarstedt, Nümbrecht, FRG, plastic granulate removed) with 25 µl Liquemin (from Hoffmann-La Roche, Basel, Switzerland, $25 \cdot 10^6$ IE/l) and the blood cells are separated by centrifugation in a centrifuge (heatable Varifuge from Heraeus-Christ, Osterode, FRG) heated to 37 °C for 5 minutes at 3500 min^{-1}. The plasma supernatant is transferred into a 20 ml disposable syringe from Braun, Melsungen, FRG and there mixed with 50 µl [^{14}C]5,5-dimethyl-2,4-oxazolidinedione solution (preparation CFA 575 from Amersham-Buchler, Braunschweig, FRG, specific activity 1.85 GBq/mmol (50 mCi/mmol), dissolved in 5 ml *Ringer*'s solution) and 50 µl of a solution of non-labelled DMO (Sigma, Deisenhofen, FRG), (5 g/l in *Ringer*'s solution). The erythrocyte sediment is freed from plasma residues and "buffy coat" by means of a water jet from glass capillary. The erythrocytes are then added again to the autologous DMO-containing plasma and thoroughly mixed (total concentration of DMO ca. 0.1 mmol/l, activity ca. 6000 disintegrations/min per 100 µl).

Incubation is carried out for 10 minutes with constant shaking in a tonometry apparatus (8) with two parallel tonometer balls, each filled with approximately 10 ml incubate and gasified with 0.06 volumes CO_2 in O_2. Using a silicone tube, the contents of the two tonometer balls are transferred, with continued gasification, into blood gas monovettes (safety monovette for the blood gas analysis, max. 4.5 ml volume, from Sarstedt Nümbrecht, FRG, two per tonometer ball); any air bubbles are expelled via the Luer opening from the monovettes, and the suspension is centrifuged at 37 °C (see above). The pH of the plasma supernatant is determined immediately after the centrifugation (extracellular pH = pH_e). After expelling the residual plasma supernatant and the uppermost erythrocyte layer mixed with plasma, a specimen of the cell sediment is taken for the haematocrit determination (see below) and the remaining cell sediment in the monovette is frozen in liquid N_2. After a minimum freezing time of 10 minutes it is thawed and refrozen, since, as phase microscopy has shown, the cells are only completely haemolysed after the second thaw. Directly after preparation of the haemolysate the pH is measured, and specimens are subsequently taken from the same material for the determination of the DMO and water contents.

Measuring technique

Measurements of pH with the micro blood system according to Astrup (BMS 2, Radiometer, Willich, FRG) at 37 °C. Calibration with calibrating buffers from Radiometer. Quality control alternatively with control specimens "Qualicheck" (Radiometer, Willich, FRG) and "Certain" (Corning, Halstead, Essex, UK). In addition the apparatus setting was checked with reference buffer solutions of the National Bureau of Standards, Washington (NBS, No. 186-I-c and 186-II-c). The precision in the series was determined as s = 0.001 (n = 25).

[^{14}C]*DMO measurement* with the liquid scintillation counter Tricarb 460 CD from Packard, Frankfurt, FRG. Preparation of specimens for scintillation counting: About 100 µl plasma or haemolysate test samples are weighed exactly in glass vials (Packard, Frankfurt, FRG) (the measurement of the volume of the sample by pipetting with an Eppendorf pipette proved too inexact because of the thick consistency of the haemolysate). The haemolysate specimens were digested with Lumasolve (from Lumac, Schaesberg, Netherlands) mixed with isopropanol (1 + 1 parts by volume), then decolorized with 0.5 ml per specimen hydrogen peroxide (perhydrol, 30%, Merck, Darmstadt, FRG). Scintillator: Lumagel (Lumac, Schaesberg, Netherlands) acidified by the addition of 0.5 mol/l hydrochloric acid (1 volume part HCl and 8 volume parts lumagel). Because of the great differences in quench between plasma and haemolysate cocktails, an internal standard was used for quench correction. The counting efficiency was determined individually for each specimen: 10 µl [^{14}C]*n*-hexadecane ($1.128 \cdot 10^6$ disintegrations/min per g from Amersham-Buchler, Braunschweig, FRG, assigned value = 8720 disintegrations/min per 10 µl) were added from a Hamilton syringe. Measurements were made at least one week after the preparation of the cocktails. Only in this manner was quench stability guaranteed between the first and second measurements of the specimens, allowing for decay of chemiluminescence and volatilization of the remaining O_2. The counting efficiency of the tests lay between 0.7 (haemolysate) and 0.9 (plasma).

Calculation of the intracellular pH value (pH_i) by using the equation (9) derived from *Jacobs* (10) (pK_a-value for DMO 6.13 (3), DMO concentrations given as disintegrations/min value per gram of haemolysate water (c_i) or plasma water (c_e)):

$$pH_i = pK_a + \log\left[\frac{c_i}{c_e}\left(1 + 10^{pH_e - pK_a}\right) - 1\right]$$

Determination of water content by weighing a 100 mg (approx.) specimen before and after treatment in the drying cabinet at 80 °C for about 20 hours, followed by drying in a desiccator over P_2O_5 to constant weight (deviation from previous weighing 0.3 mg maximum).

Haematrocrit determination: Withdrawal of the cell material after the centrifugation: a microhaematocrit capillary is filled via the Luer opening of the monovette. Centrifugation: Hemofuge from Heraeus-Christ, Osterode, FRG, with 12000 min^{-1}. The packed cells had an average haematocrit of 0.96 (s = 0.0063, n = 16).

Results

The data and measurement results of a total of 16 tests on blood specimens of healthy donors are summarized in table 1. The pH values and all further measurement data are average values of 4 measurements on 4 subspecimens (in each case 2 tests from 1 of the 2 tonometer fillings of the same blood specimen). Due to the standardized gasification, the pH values were all in the reference interval for arterial blood (pH between 7.37 and 7.45). The values for the intracellular pH (pH_i (potentiometric methods) and pH_i (DMO method)) were lower than the associated pH_e values, in accordance with the stronger acid environment in the erythrocytes. The two methods produced comparable results for the pH_i. However, the results of the potentiometric measurements were regularly slightly higher than those of the DMO method (see tab. 1). The average value of the difference between pH_i (potentiometric method) and pH_i (DMO method) was 0.027 with a standard deviation of 0.012.

Tab. 1. Results of the determination of intracellular pH in human erythrocytes. Potentiometric direct determination in haemolysate, and the DMO method applied to the same specimen

Test No.	pH_e	Water Content*)		Concentration quotient c_i/c_e	pH_i		Difference pH_i(potent.)-pH_i(DMO)
		Haemolysate	Plasma		DMO	potent.	
1	7.414	0.662	0.921	0.585	7.165	7.191	0.026
2	7.379	0.662	0.911	0.597	7.138	7.147	0.009
3	7.418	0.668	0.905	0.578	7.163	7.194	0.031
4	7.424	0.670	0.910	0.590	7.179	7.200	0.021
5	7.400	0.664	0.916	0.591	7.155	7.184	0.029
6	7.418	0.663	0.916	0.590	7.173	7.206	0.033
7	7.450	0.660	0.918	0.575	7.194	7.207	0.013
8	7.375	0.660	0.908	0.568	7.110	7.141	0.031
9	7.383	0.653	0.909	0.562	7.113	7.148	0.035
10	7.402	0.666	0.911	0.583	7.150	7.188	0.038
11	7.397	0.664	0.911	0.572	7.136	7.187	0.051
12	7.404	0.666	0.913	0.578	7.149	7.186	0.037
13	7.420	0.682	0.922	0.600	7.183	7.191	0.008
14	7.410	0.679	0.919	0.593	7.167	7.181	0.014
15	7.383	0.669	0.919	0.594	7.140	7.179	0.039
16	7.374	0.677	0.921	0.625	7.155	7.169	0.014
$\bar{x}$		0.667	0.914				0.027
s		0.0075	0.0053				0.012

*) mass fraction

The difference between the average values of the two methods is statistically significant ($p < 0.001$, paired t-test). The slope of the standardized principal component model (see tab. 2) was however not significantly different from 1. Whereas occasionally the pH_i values of the two different methods are almost identical (differences of 0.01 pH units), the deviations in the extreme case are 0.05 pH units. An explanation for these differences cannot be deduced from the measurement results: The measured quantities correlate only poorly (concentrations quotient c_i/c_e or pH gradient $pH_e - pH_i$) or not at all (water content of haemolysate and plasma) with the pH_i differences of the two methods. As expected, the pH_i values of both methods show a dependence on pH_e, and this dependence is linear (correlation coefficient of the linear regression for pH_i (DMO method) $r = 0.86$ and for pH_i (potentiometric method) $r = 0.83$).

Tab. 2. Statistical data for comparison of the methods.

	pH_i DMO-method	pH_i potentiometric method
Mean value	7.154	7.181
Stand. principal component model		
intercept		1.012
slope		0.86
Standard error of residues	$s_{y.x}$	0.008
Correlation coefficient linear regression	r	0.85

Discussion

After careful optimization of the specimen preparation and of the analytical procedures, the intracellular pH values in human erythrocytes determined with the two methods under examination showed only a low, albeit statistically verified average difference of 0.027 pH units. Larger differences are reported in the literature (6, 7, 11). The differences found in the present investigations vary with the individual tests, but show systematically higher pH_i values with the potentiometric measurement than with the DMO method. This raises the question of the cause of this systematic deviation.

In the potentiometric method, the haemolysate is contaminated by the trapped plasma in the intercellular space. For a mean pH difference ($pH_e - pH_i$) of 0.22 and a haematocrit value of 0.96, the increase of the pH value due to the plasma contamination is calculated to be less than 0.001 of a pH unit. This value is in agreement with figures in the literature (12) and lies at the limit of the sensitivity of the pH measurement.

In own results, plasma contamination is not a possible source of error, because the DMO method was applied to the same specimen material, likewise without making allowance for any plasma contamination. A mathematical correction for the plasma contamination in the DMO method would result in a somewhat greater effect because of the higher pH difference ($pH_e - pH_i$) (mean value 0.25 compared with 0.22 in the potentiometric method).

Other interference factors in the potentiometric method are:

1) the so-called "liquid junction potential" (13),
2) glucose breakdown by glycolysis (14) or via the hexose monophosphate shunt (15),
3) a CO_2 shift during the centrifugation (if the temperature of 37 °C is not maintained) (13).

These interference factors cannot always be ruled out with certainty. However, they do not provide an explanation for the observed discrepancies between the two techniques, since they would reduce the pH_i values, whereas the potentiometrically determined pH_i values are larger than in the DMO method. Interference factors possibly increasing the pH_i are as follows:

1) Losses of O_2 or CO_2 during the specimen preparation (16). In order to avoid a gas exchange of this sort, the tests were carried out under anaerobic conditions in gas-tight collecting tubes.

2) The apparent pH_i could also be increased by contamination of the liquid junction blood/KCl (17). To avoid such contamination, the KCl bridge was rinsed and replaced after each measurement series.

Consequently there was no apparent reason for erroneous results in the pH determination with the potentiometric method.

An analysis of the errors of the DMO method was made easier by being able to check for and eliminate errors of a fundamental nature, e. g. permeability of the erythrocyte membrane with respect to the dissociated DMO component (1), physicochemical inhomogeneities through intracellular compartmentalization (18), and a binding of the DMO to plasma (19) and erythrocyte proteins, in particular haemoglobin (12). It can also be assumed that the pK_a values for DMO in plasma and cells agree. The ion strengths of serum and erythrocytes differ so little (20) that they give rise to only insignificant differences in the pK_a values (*Waddell & Butler* (3)). Thus the theoretical preconditions for correct measurement are fulfilled. With the great number of separate determinations which are required in the DMO method, the possibility of experimentally induced systemic errors is, however, particularly large, as *Robson* et al. (21) have already pointed out. The pH_i values determined with the DMO method are largely governed by the quotient of the DMO concentrations in cells (c_i) and plasma (c_e). It is therefore obvious to look for error sources in its formulation. The concentration figures for the DMO must be related to the water space in which the DMO is distributed. In our calculations, according normal practice, the analysed DMO amount (measured as disintegrations/min per g specimen) was related to the total water in plasma or haemolysate (here, for reasons of comparison without deduction of the plasma water, which makes up about 0.04 of the haemolysate). In order to detect test-specific fluctuations of the water content (in the erythrocyte a dependence of water content on pH_e (22, 23) exists, known as *Gibbs-Donnan* effect), the latter was determined for each test.

In spite of this, errors can arise in relating the DMO amount to the water content of the specimen. It is by no means sure that the water from plasma and cells is completely available as a solution space. On the contrary, results are available which throw doubt on this, at least for erythrocytes, where part of the cell water is bound by hydration, in particular by haemoglobin (24, 25). It is precisely with erythrocytes, which have a relatively low water content, that even slightly incorrect estimations of the water space would have considerable influence on calculated concentrations. If, as a result of the presence of bonded water, the osmotically active water space is less than the water content, then the calculated DMO concentrations in the erythrocytes, the resulting concentration quotient and the pH_i values derived from them will be too low.

In our investigations the DMO method does in fact furnish values which are lower than those from the potentiometric method. An overestimation of the water phase with the DMO method may thus be the reason for differences observed between the two methods. Theoretically, such a bias should regularly lead to the same deviation. With the complexity of other factors which influence the results of the two methods to a greater or lesser degree, and can superimpose themselves on this effect, a constant deviation can hardly be expected.

On the basis of these considerations, the DMO method for checking the direct potentiometric determination of the pH value in erythrocytes seems to be advisable only if it is possible to define precisely the water phase of the erythrocytes as solution space. On the other hand, the deviation obtained after the optimization of the methods is so small that both methods appear to be sufficiently reliable for practical purposes. Because of its simple implementation, the direct potentiometric pH determination of haemolysate is the more suitable method for clinical purposes.

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